AWARD NUMBER: W81XWH-15-1-0340

TITLE: Vagus Nerve Stimulation as a Treatment Strategy for Gulf War Illness

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REPORT DATE: October 2016

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
October 2016	Annual	30 Sep 2015 - 29 Sep 2016

4. TITLE AND SUBTITLE

Vagus Nerve Stimulation as a Treatment Strategy for Gulf War Illness

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-15-1-0340

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
TEXAS A&M UNIVERSITY SYSTEM, THE
200 TECHNOLOGY WAY
STE 2079
COLLEGE STATION TX 77845-3424

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

The purpose of this research is to determine if vagus nerve stimulation (VNS) will be an effective therapeutic strategy for Gulf War Illness (GWI). GWI refers to a chronic complex of symptoms observed in afflicted personnel. GWI symptoms include cognitive impairments (memory and concentration problems), headaches, migraines, widespread pain, fatigue, gastrointestinal and respiratory issues, as well as other unexplained abnormalities that do not fit into classical medical diagnostic criteria. There are extensive clinical and experimental data showing that VNS treatment exerts beneficial effects in many of the aforementioned symptom domains associated with GWI. We are in the process of testing the efficacy of VNS treatment on behavioral, cognitive, inflammatory, neuroinflammatory and neuroanatomical outcome measures.

15. SUBJECT TERMS
Permethrin, pyridostigmine bromide, inflammation, neuroinflammation, astrocyte activation, cholinergic anti-inflammatory pathway

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified	11	19b. TELEPHONE NUMBER *(include area code)*

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1. **INTRODUCTION:**

The purpose of this research is to determine if vagus nerve stimulation (VNS) will be an effective therapeutic strategy for Gulf War Illness (GWI). GWI refers to a chronic complex of symptoms observed in afflicted personnel. GWI symptoms include cognitive impairments (memory and concentration problems), headaches, migraines, widespread pain, fatigue, gastrointestinal and respiratory issues, as well as other unexplained abnormalities that do not fit into classical medical diagnostic criteria. There are extensive clinical and experimental data showing that VNS treatment exerts beneficial effects in many of the aforementioned symptom domains associated with GWI. Therefore, using an established animal model of GWI, we will test the efficacy of vagus nerve stimulation, initiated at a time-point analogous to >20 years after the initial exposure to GWI compounds, on cognitive, behavioral, inflammatory, neuroinflammatory, and neuroanatomical outcomes.

2. **KEYWORDS:** Permethrin, pyridostigmine bromide, inflammation, neuroinflammation, astrocyte activation, cholinergic anti-inflammatory pathway

3. **ACCOMPLISHMENTS:**

What were the major goals of the project?

Thee major goals of the project as stated in the approved SOW were to perform the analysis on a total of 60 mice, as described below.

Year 1 60 mice (30 from Aim 1 and 30 from Aim 2) 6 mice from each group. Groups are as follows:

Specific Aim 1, Year 1. List of groups and mice per group	N
Group 1: Naïve mice	6
Group 2: GWI controls (exposed to chemicals only, no further manipulations	6
Group 3: Vehicle treated controls (exposed to saline + diluting agent (DMSO), no further manipulations)	6
Group 4: GWI animals (exposed to chemicals) implanted with VNS stimulator, stimulators not turned on	6
Group 5: GWI animals (exposed to chemicals) implanted with VNS stimulator and stimulators turned on	6

Specific Aim 2, Year 1. List of groups and mice per group	N
Group 1: Naïve mice	6
Group 2: GWI controls (exposed to chemicals only, no further manipulations	6
Group 3: Vehicle treated controls (exposed to saline + diluting agent (DMSO), no further manipulations)	6
Group 4: GWI animals (exposed to chemicals) implanted with VNS stimulator, stimulators not turned on	6
Group 5: GWI animals (exposed to chemicals) implanted with VNS stimulator and stimulators turned on	6

In each case, the mice are exposed to GWI chemicals at experimental days 1-10. Then, the animals receive standard care, for ~ 220 days, after which they are tested on the von frey pain test pre-test. 3-5 days after this behavior testing, mice in the vagus nerve stimulator implantation groups undergo this procedure. Then, after a

2- to 5-day recovery time, the VNS stimulators are turned on for either 2 weeks (Aim 1), or 4 weeks (Aim 2). After the completion of the stimulation paradigm, the following behavioral tests occur:

Task order	Task type	Task Duration
1	Von Frey pain threshold post-test	1 day followed by 3 days of rest
2	Open field test	1 day followed by 3 days of rest
3	Object location test	1 day followed by 3 days of rest
4	Novel object recognition test	1 day followed by 3 days of rest
5	Morris water maze test	7 day learning + 1 day probe test and visible platform test

After these behavioral tasks, mice are sacrificed for anatomical and biological analysis.

What was accomplished under these goals?

1) **Major activities**: We organized the studies such that we purchase 10 mice at a time, and within every 10 mice ordered, 2 mice are randomly assigned to each of the 5 groups in Aim 1 or 2. In total, during year 1, we have purchased a total of 160 mice. Of these, 96 mice have been injected with the Gulf War chemicals, 32 have been injected with DMSO (vehicle controls), and 32 were in the naïve group. We have removed a total of 28 mice from the experiment. These mice include Naïve (2.5%), DMSO (2.5%), GWI (10.6%), and GWI mice implanted with the VNS stimulators (1.9%). The mice have been removed for: mortality, fighting or other wounds that could not be adequately treated without compromising the variables, surgical implantation failure.

   In total, 60 mice were on schedule to complete all of the tasks, including behavioral analysis, specified in the experimental design. As a result of mortality or removal from the study, 49 of the 60 have thus far completed the entirety of the studies. We are further in the behavioral testing stage for an additional 16 mice, and the completion dates of the behavioral battery for all of these mice is between October 21st and November 4th. Thus, we will have completed a total of 65 mice at right around the 1-year point of the funding.

   In addition, using outside funds, we confirmed the efficacy of our implementation of the GWI model, using a group of 4 GWI mice, 4 DMSO mice and 2 Naïve mice. 1 GWI mouse and 1 DMSO mouse did not survive to be tested behaviorally (fighting wounds). Despite being under-powered, we performed behavioral testing between 3 and 5 months after the induction of the GWI chemicals (or DMSO). These time points were selected because Dr. Crawford, the originator of this model, has previously demonstrated behavioral/cognitive impairments at these time points. In performing these preliminary experiments, it also enabled us the opportunity to completely work out our behavioral protocols, on collaboration with Dr. Shetty. Despite being under-powered, our results confirmed the previous studies from Dr. Crawford, showing trends (object location task and pattern separation task) or significant impairments (open field, Von Frey pain test), in our behavioral tasks, and confirmed our ability to implement the GWI model, as well as the behavioral testing.

2) **Specific Objectives**: Above and beyond the group of mice that we paid for using our other lab funds, all of the mice used as part of the grant have met the specific objectives as specified in the statement of work.

3) **Significant Results**: Notably, we have observed an increase pain sensitivity in the GWI mice, in our preliminary test performed using our other lab funds. Because this particular study is under-powered, we are still discussing whether or not the data are publishable in their current form.

4) **Other achievements and goals not met**: In the group of mice that were assessed using other funds, we performed flow cytometry on the spleens and intestines of these mice. We found evidence of splenocyte activation, as well as activation and expansion of major histocompatibility complex (MHC) II-expressing B cells (**Fig. 1**).

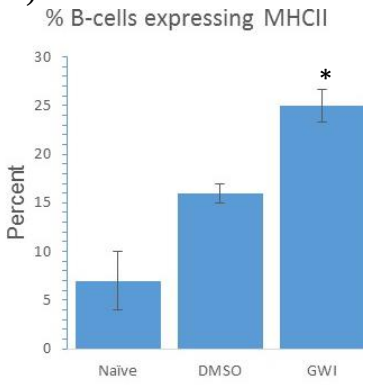

Figure 1. Percent of B cells in the spleen that also express MHCII. An increase in MHCII expression on B cells is indicative of T cell-dependent activation of the B cells. As can be seen in the graph, spleens harvested at the outset of behavioral testing have significantly more MHCII+ B cells in GWI mice, compared to DMSO or Naïve mice. It is pertinent to note that a trend toward an increase was observed in DMSO mice compared to Naïve, but this result was not significant.

Further evidence in support of the activation of B cells in the GWI mice is observed by staining the splenocytes for immunoglobulin D **(Fig. 2)**.

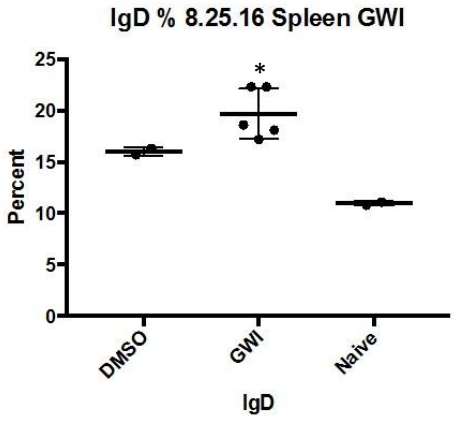

Figure 2. Flow cytometric analysis of immunoglobulin D (IgD) expression in splenocytes. IgD is an immunoglobulin that appears in species with an adaptive immune system. Among its numerous activities in the adaptive immune response, IgD is involved in B cell activation. As can be seen in the graph, IgD is significantly increased in GWI mice compared to DMSO mice and naïve mice. It is pertinent to note that we also examined IgM, but did not detect any significant differences).

It should be noted that we have also examined, using flow cytometry, a number of other markers of lymphocyte activation, adaptive immune response, and memory immune cells (Not shown). Although none of the results were significant, there were some trends, and considering that at the time of this writing, such analysis is underpowered, using our own funds, we will continue to perform this analysis on spleens, as our resources allow. We expect that this will allow us to define some of the cellular mediators in the immune response to our model of GWI, and possibly identify therapeutic targets in the future. It should be further noted, that in consideration of gastrointestinal (GI) issues with GWI patients, we have also added flow cytometric analysis of the intestines to our protocols. As with the spleens, this analysis will be done with money from the PI's lab that is separate from the grant. While we have performed some analysis of this type, the we have N's of less than 3 for all groups, and therefore the data are not ready to be presented. We look forward to having this data to present for our next progress report.

In addition, we have added a behavior to our behavioral testing battery, the pattern separation task. The addition of this behavior does not alter the existing order and timing of the behaviors, because we are able to obtain the open field data from the first trial of the object location task. The advantage of adding the pattern separation task is that it allows us to further identify specific neuroanatomical substrates for any deficits/therapeutic improvements. In the case of the pattern separation task, it allows us to examine functional correlates of adult neurogenesis and dentate gyrus circuitry.

Thus far, we are on target to achieve all of the goals of our grant. However, because of the amount of time it took us to get all of the experiments fully underway, as well as our desire to perform the analysis on groups of equally matched experimental conditions, we have decided to push the dendritic analysis off until year 2, when we will be able to provide enough brains from each condition to satisfy our power analysis requirements (N = 6 per group). As such, we expect to see results from this analysis in year 2.

What opportunities for training and professional development has the project provided?

Although this project was not intended to provide training and professional development opportunities, it has presented an excellent opportunity to train an up-and-coming scientist (Dr. Damir Nizamutdinov), on the rigors of carrying-out experiments as they are intended in a grant proposal, as well as the importance of being highly-organized, such that all of the data are optimally useful. Along these lines, Damir has also gained expertise in a series of surgical, behavioral and neuroanatomical techniques, as well as significant one-on-one time with a mentor (Dr. Shapiro). Damir will also attend the annual Society for Neuroscience Conference this year, in which he will be able to enhance his professional development.

In addition, we are pleased that this funding has provided a training opportunity for a volunteer in the lab. Jaclyn Jenkins, an Army Veteran, has been undergoing a number of training activities in my lab. The initial idea for the training was to expose Jaclyn to the lab setting, and enhance her skill set, giving her a number of opportunities that include: 1) Working as a technician in the future; 2) Pursuing higher education (BS, MA); 3) Pursuing a Ph.D.; 4) Pursuing an MD. While all of the options remain in play, currently Jaclyn has demonstrated a high capacity for laboratory work, and a growing interest in doing so. Although Jaclyn is currently enrolled in an Associate degree program, she is already planning her enrollment in a 4 year college, with an eye toward either a Ph.D., or an MD, further down the road. It needs to be emphasized that the ability to work on a project that involves exposure to GWI chemicals (some of which she was also exposed to in her military service) was the initial impetus for her interest. We are honored to be able to facilitate Jaclyn's growing interests!

How were the results disseminated to communities of interest?

Although we do not yet have results that are directly specified by the grant, I've discussed, at great length, our progress on the proposal, as well as our findings above and beyond those in the proposal. These discussions include weekly and/or bi-weekly meetings with Dr. Shetty, a Co-I on the grant. In addition, the decision to use our other lab funds to ensure that we have properly implemented the model was done in communication with Lea Steele. Dr. Steele also suggested that we perform several other tests on this group of mice. Additionally, through Dr. Steele's enormous network of GWI researches, she has put me in touch with a group in New York who are performing a small scale human trial of vagus nerve stimulation on GWI patients. We intend to keep the lines of communication open, such that the findings from the respective studies can be placed in the optimal context. Finally, I have had the opportunity to discuss our progress with other experts in the field, whom I met while serving on the GWI grant review panel.

What do you plan to do during the next reporting period to accomplish the goals?

We expect to continue as planned, and fully intend to accomplish the goals set forth, as well as pursuing other highly intriguing avenues of GWI research.

Specifically, as specified in the grant, we will complete the analysis of an additional 90 mice, as below:

For years 2 and 3, the organization of the studies will be the same as above. The only differences will be in the number of mice that we test. These numbers are as follows:

Specific Aim 1, Year 2. List of groups and mice per group	N
Group 1: Naïve mice	9
Group 2: GWI controls (exposed to chemicals only, no further manipulations	9
Group 3: Vehicle treated controls (exposed to saline + diluting agent (DMSO), no further manipulations)	9
Group 4: GWI animals (exposed to chemicals) implanted with VNS stimulator, stimulators not turned on	9
Group 5: GWI animals (exposed to chemicals) implanted with VNS stimulator and stimulators turned on	9

Specific Aim 2, Year 2. List of groups and mice per group	N
Group 1: Naïve mice	9
Group 2: GWI controls (exposed to chemicals only, no further manipulations	9
Group 3: Vehicle treated controls (exposed to saline + diluting agent (DMSO), no further manipulations)	9
Group 4: GWI animals (exposed to chemicals) implanted with VNS stimulator, stimulators not turned on	9
Group 5: GWI animals (exposed to chemicals) implanted with VNS stimulator and stimulators turned on	9

We expect to complete analysis of these 90 mice. It should be noted that we have already initiated the injection protocol for 72 (surviving) of the 90 mice to be used in year two. These mice will go through the behavioral, anatomical, and molecular analysis during year 2. We will also add an additional 2 to 3 groups of 10 mice in year 2, so that we analyze at least the 90 specified in the proposal, and likely more. These latter groups of mice will be injected beginning in November, 2016. In addition, based on our experiences during year 1, it will be necessary during year 2, to initiate the GWI injection protocol on the 60 mice that will be analyzed in year 3. We expect to initiate these injections beginning mid-2017. This will ensure that we are able to collect all of the data from these mice in year 3, and still have time to complete the analysis.

4. **IMPACT:** *Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:*

What was the impact on the development of the principal discipline(s) of the project?

We have now optimized the model of Gulf war illness, and also obtained expertise in isolating the vagus nerve, and implanting the stimulator around the nerve.

What was the impact on other disciplines?

Based on our ability, and now expertise, at implanting the vagus nerve stimulators, as well as the collaboration with Dr. Stauss, we have recently submitted an NIH R21 proposal. We propose to assess the potential of vagus nerve stimulation on treating diabetes. This collaborative effort would never had happened, had this current GWI proposal not been funded.

What was the impact on technology transfer?

Nothing to Report.

What was the impact on society beyond science and technology?

Nothing to Report. However, it is pertinent to note that during my grant review responsibilities, I had a number of very positive interactions with GWI Veterans, and all were highly enthusiastic about the possibilities represented by our studies.

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change

The only change to the approach is that we have initiated the model in more mice during the first year than originally planned. The reason for this was due to the delay in hiring the requisite staff, which set-us behind schedule. In order to make sure that the proposed experiments are completed within the 3-year funding period, we have initiated the GWI protocol in more groups starting in year 1. Because our protocol requires a lengthy time in the study (~ 9 months per subject), it was necessary to initiate these models earlier than expected. It should be further noted, that although we have already initiated the GWI injection paradigm in these mice, in year 1, the behavioral, anatomical and molecular analysis will not take place until year 2.

Actual or anticipated problems or delays and actions or plans to resolve them

As above, the major delay that we encountered was related to the time it took to identify the ideal candidate, then to subsequently go through the hiring process, after which a training period was necessary. As above, we have added more groups of mice to year 1, and this will ultimately put us back on track to complete the proposed studies in the 3-year period of the grant.

Changes that had a significant impact on expenditures

Consistent with the above delay in hiring staff, we will have approximately 10-25% of leftover funds. We plan to spend them in the coming years, as we catch up on this delay.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

The internal approval dates for our protocol are: 7/28/2015 expires 7/28/2018

The DoD/US Army approval dates for our protocol are: 10/21/2015

We've adhered strictly to the approved protocol, and do not have any changes to report at this time.

6. PRODUCTS: Nothing to Report

Publications, conference papers, and presentations

Journal publications. Nothing to report

Books or other non-periodical, one-time publications. Nothing to report

Other publications, conference papers, and presentations. Nothing to report.

Website(s) or other Internet site(s). Nothing to report

Technologies or techniques. Nothing to report

Inventions, patent applications, and/or licenses. Nothing to report

Other Products. Together with our collaborators, we've identified a role of vagus nerve stimulation in regulating glucose metabolism. We are in the process of drafting an R21 proposal to further investigate the therapeutic potential of this finding.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	*Lee A. Shapiro*
Project Role:	*PI (No change)*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	
Contribution to Project:	*No change*
Funding Support:	*Internal lab funds (to validate model and perform flow cytometry above and beyond funded project).*

Name:	*Ashok Shetty*
Project Role:	*No change*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	
Contribution to Project:	*No change*
Funding Support:	

Name:	*Harald Stauss*
Project Role:	*No change*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	
Contribution to Project:	*No change*
Funding Support:	

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to Report.

What other organizations were involved as partners?

- **Organization Name:** University of Iowa
- **Location of Organization:** *Iowa, USA*
- **Partner's contribution to the projec**t. Dr. Harald Stuass; manufactures the custom vagus nerve stimulators. Harald also came to TX to demonstrate how to implant the stimulator coils around the carotid sheath, and also how to place the stimulator in the subcutaneous space.
  - **Financial support;** *Co-I on grant, U of Iowa funds*
  - **In-kind support** *Partner makes the vagus nerve stimulators*
  - **Facilities** *NA*
  - **Collaboration** *Dr. Stauss visited our lab in March, 2016, in order to instruct us on the proper implantation of the vagus nerve stimulators, as well as the correct way to ensure proper activation/de-activation of the stimulators.*
  - **Personnel exchanges** *N/A*
  - **Other.** *All work with Dr. Stauss occurred as specified in the proposal.*

8. **SPECIAL REPORTING REQUIREMENTS:** None
9. **APPENDICES:** *Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc. Reminder: Pages shall be consecutively numbered throughout the report.* ***DO NOT RENUMBER PAGES IN THE APPENDICES.***